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Cytotoxic Effect of Trace Metals Adsorbed Onto Fly Ash Particles

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16. ABSTRACT

Fly ash particles fractionated into three size ranges (<2, 2-5 and 5-8μ) were coated with lead, nickel and manganese oxides to determine the effects of particle size and surface area on the in vitro toxicity of these metals for alveolar macrophages. When uncoated or coated fly ash particles in concentrations ranging from 50 to 1500 μg/ml were incubated with 10^6AM/ml, reduced viability and increased phagocytosis were observed upon increase in concentration. This was seen irrespective of the particle size. However, within a given particle coating and concentration, decrease in particle size resulted in decreased viability and increased phagocytosis. The percentage of lead, nickel or manganese adsorbed on the fly ash particles varied within a relatively narrow range, and was not affected by the particle size. Since the macrophages were exposed to the particles on a weight-dose-per-cell basis, the concentration of metal oxides to which they were exposed was constant, irrespective of the particle size. The greater toxicity of the small particles appeared to be due to surface interaction between particles and the alveolar macrophages. Thus, the toxic effect is surface-area as well as dose related. These observations were further supported by decreases in total cellular protein levels and specific activity of LDH.

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CYTOTOXIC EFFECT OF TRACE METALS ADSORBED ONTO FLY ASH PARTICLES

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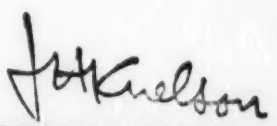
FOREWORD

The many benefits of our modern, developing, industrial society are accompanied by certain hazards. Careful assessment of the relative risk of existing and new man-made environmental hazards is necessary for the establishment of sound regulatory policy. These regulations serve to enhance the quality of our environment in order to promote the public health and welfare and the productive capacity of our Nation's population.

The Health Effects Research Laboratory, Research Triangle Park, conducts a coordinated environmental health research program in toxicology, epidemiology, and clinical studies using human volunteer subjects. These studies address problems in air pollution, non-ionizing radiation, environmental carcinogenesis and the toxicology of pesticides as well as other chemical pollutants. The Laboratory develops and revises air quality criteria documents on pollutants for which national ambient air quality standards exist or are proposed, provides the data for registration of new pesticides or proposed suspension of those already in use, conducts research on hazardous and toxic materials, and is preparing the health basis for non-ionizing radiation standards. Direct support to the regulatory function of the Agency is provided in the form of expert testimony and preparation of affidavits as well as expert advice to the Administrator to assure the adequacy of health care and surveillance of persons having suffered imminent and substantial endangerment of their health.

This report describes the results of an *in vitro* study designed to determine the effects of fly ash coated with the oxides of lead, nickel or manganese on alveolar macrophages which defend the deep lung against infectious microorganisms. Since particulates of various sizes exist within the environment, the influence of particle size was also examined in order to provide data helpful in designing pollutant control strategies. It was found that as the concentration of pollutant was increased, the toxicity increased. In addition, the smaller-sized particles were more toxic than larger particulates.



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ABSTRACT

Fly ash particles fractionated into three size ranges (<2, 2-5 and 5-8μ) were coated with lead, nickel and manganese oxides to determine the effects of particle size and surface area on the *in vitro* toxicity of these metals for alveolar macrophages. When uncoated or coated fly ash particles in concentrations ranging from 50 to 1500 μg/ml were incubated with 10^6AM/ml, reduced viability and increased phagocytosis were observed upon increase in concentration. This was seen irrespective of the particle size. However, within a given particle coating and concentration, decrease in particle size resulted in decreased viability and increased phagocytosis. The percentage of lead, nickel or manganese adsorbed on the fly ash particles varied within a relatively narrow range, and was not affected by the particle size. Since the macrophages were exposed to the particles on a weight-dose-per-cell basis, the concentration of metal oxides to which they were exposed was constant, irrespective of the particle size. The greater toxicity of the small particles appeared to be due to surface interaction between particles and the alveolar macrophages. Thus, the toxic effect is surface-area as well as dose related. These observations were further supported by decreases in total cellular protein levels and specific activity of LDH.

This report was submitted in fulfillment of Contract 68-02-0761 by IIT Research Institute under the sponsorship of the U.S. Environmental Protection Agency. This report covers the period from April 1975 to September 1976 and work was completed as of October 31, 1976.

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SECTION 1

INTRODUCTION

The overall objective of this project was to examine *in vivo* and *in vitro* effects of various air pollutants on pulmonary alveolar macrophages. During the first two years the effects of inhalation of nitrogen dioxide (NO_2) and ozone (O_3) on mice were investigated. The observations included total and differential cell counts, viability, cell surface morphology, and *in vitro* phagocytic function of the alveolar macrophages obtained by tracheobronchial lavage from mice exposed to the pollutants. The changes in the bactericidal capacity of alveolar macrophages in lungs of mice challenged, by the respiratory route, with *Staphylococcus aureus* after exposure to the air pollutants were determined. In addition, the surface morphologic alterations observed in alveolar macrophages lavaged from the lungs and the emphysemateous changes in the alveolar and terminal airway structure of the lungs of animals exposed to NO2 were studied by scanning electron microscopy. These studies have been summarized in annual reports IITRI Report L6070-4 (1974) and IITRI Report L6070-8 (1975). In another phase of the program, studies were conducted on the exposure of guinea pigs to NO_2 (IITRI Report L6070-10P (1975)).

During the year covered by this report, studies were carried out to correlate the toxicity for normal rabbit alveolar macrophages of lead, nickel and manganese oxides with the physical characteristics of fly ash particles. The toxicity of the metal oxides as affected by the particle size and surface area of the carrier fly ash on which they had been adsorbed was investigated. The parameters of toxicity were changes in alveolar macrophage viability, cell lysis and activity of selected cellular enzymes.

SECTION 2

CONCLUSIONS

Various concentrations of fly ash separated into <2, 2 to 5 and 5 to 8μ particle size ranges and coated with lead, nickel or manganese oxide were incubated for 21 hr with normal rabbit alveolar macrophages. Changes in viability, total cellular protein content and specific enzyme activities were monitored. A linear dose response relationship between concentration and cell viability, total cellular protein levels and LDH specific activity was observed for all particles. The levels of the lysosomal enzymes acid phosphatase and β-glucuronidase showed, generally, no change. The results provided a toxic ranking for the metal oxides, i.e., Pb >Ni ≥Mn and demonstrated that toxicity was not due to solubilization of the metal compounds in the incubating medium. For a given metal oxide and a given particle concentration, toxic effects increased with decreasing particle size. Since the macrophages were exposed to the particles on a weight concentration per cell number basis and, moreover, the percentage of Pb, Ni or Mn, was approximately the same for all particle sizes, the concentration of the metals was not affected by the particle size. Therefore, the greater toxicity of the smaller particles appeared to be due to their larger surface area. Consequently, the particle itself played a role in the toxicity, serving as a carrier of the metal compounds into the intracellular millieu.

The stability of the specific activity of the lysosomal hydrolases (acid phosphatase, β-glucuronidase) as compared to that of a soluble enzyme in the cytosol (LDH) suggests that the release of hydrolytic enzymes in the cell may be a secondary event in the cytotoxic response.

SECTION 3

RECOMMENDATIONS

Viability of alveolar macrophages appears to be the parameter of choice in establishing toxic ranking of particulate pollutants. Although it would be expected that the cellular enzyme activities should characterize the state of cellular injury more precisely and with higher sensitivity, the simplicity of the viability assay is an overriding advantage for screening studies.

The studies provide an experimental evidence that the physical nature of the particle is extremely relevant to its toxicity by demonstrating that the size of a carrier particle affects the toxicity to macrophages of metal oxides adsorbed on its surface. To explore further how the shape of a particle is involved, fibrous material of known toxicity (e.g., asbestos) should be examined relative to an inert material with similar geometric properties (e.g., glass fibers). An interesting variable to follow would be the effect on toxicity of simultaneously changing size and shape of the fibers by varying the length to width ratio in the fiber axis.

To learn more about the toxic mechanism it would be important to determine the time course of the processes. Macrophages should be incubated with the particles in concentrations that produce a 50% viability decrease in a given period and the changes in viability, activity of selected enzymes and levels of ATP for monitoring functional impairment should be determined at various time intervals. Particles containing nonsoluble toxic compounds and particles from which a toxic compound is partially solubilized during incubation should be included. The time course studies would demonstrate differences in the nature of the toxic effects on macrophages of different compounds. Most importantly the studies would provide means of comparison between the mechanism of action of a soluble and nonsoluble toxic substance on macrophages. If solubilized the compound can directly interfere with metabolic processes in the cytoplasm by permeating the plasma membrane; if nonsoluble and bound to the particle by acting after engulfment of the toxic particle in the phagolysosomes.

SECTION 4

MATERIALS AND METHODS

ANIMALS

Male albino New Zealand rabbits weighing 1.5 to 2 kg were used to obtain high yields of alveolar macrophages (AM). The rabbits were quarantined for 2 weeks before use and were provided food and water *ad libitum*.

PREPARATION OF TEST PARTICLES

The particles used in the experiments were prepared by the Fine Particles Section of IITRI from a fly ash sample provided by EPA. The fly ash was fractionated by air classification (Bahco Microparticle Classifier) into three particle size ranges, namely <2, 2 to 5 and 5 to 8μ. The metals in form of their hydroxides were then precipitated onto the surface of the fly ash particles dispersed in an aqueous phase. After filtration and washing, the particles were heated to 675°C to convert the metal hydroxides into the respective oxides (1).

HARVESTING AND CHARACTERIZATION OF AM

The rabbits were killed by injection of sodium pentobarbital into the marginal ear vein and the lungs were lavaged *in situ* with warm (37°C) sterile saline through a catheter inserted into an incision in the trachea (2-3). The AM were isolated from the lavage fluid by centrifugation at 365xg for 10 min and washing in Hanks salt solution. Total cell counts were made in a hemocytometer (3), and viability was determined by the trypan-blue dye exclusion technique (4). For differential counts, smears of air-dried cells were fixed in methanol and stained with Wright's stain.

EXPOSURE OF AM TO PARTICULATES

Medium 199 in Hanks balanced salt solution was used for incubation and exposure of the AM to the particulates. Supplements in the maintenance medium included 0.25 M Hepes buffer to further stabilize the pH (7.2 - 7.4), 100 μg/ml Gentamicin (Schering Corp.) and 10% heat-inactivated fetal calf serum. Separate AM and particle suspensions were prepared in the medium at twice the projected exposure concentrations and then equal volumes of the

two suspensions were mixed (Serum was omitted from the particle suspension to prevent coating of the particles and was added at 20% concentration to the cell suspension.) The final AM concentration in the suspensions was maintained constant at 10^6AM/ml, whereas the concentration of particles ranged from 50 to 1500 µg/10^6AM/ml medium. The suspensions were incubated in wells of disposable plastic cluster dishes placed on a rocker platform for continuous agitation for 21 hr at 37°C in a humidified 4% CO_2 atmosphere.

VIABILITY AND PHAGOCYTOSIS

After incubation, the suspensions were withdrawn from the cluster dishes with Pasteur pipettes and transferred into siliconized test tubes. The AM attached to the cluster dishes were removed by treatment with 0.25% trypsin or by use of rubber policemen, and were combined with the corresponding suspensions in the test tubes and chilled. Viability was determined by microscopic counting of 400 to 500 AM and the percentage of AM that had phagocytized was determined on the same slides.

SCREENING OF PARTICLES FOR SOLUBLE CYTOTOXIC COMPONENTS

The fly ash particles coated with the various metal oxides were incubated in the culture medium under similar conditions to those used in the experiments, with the omission of AM and serum. After incubation, the particles were removed from the media by centrifugation and were resuspended in fresh medium. The supernatant was filtered through a 0.22µ Millipore filter. The filtered supernatant (S) and the resuspended particles (P) and the original suspension (P + S) were then separately tested for cytotoxicity using the AM viability assay.

ENZYME ACTIVITY

For determination of enzyme activity, the AM were first mixed with the particles in siliconized Erlenmeyer flasks, then 10 ml of the test suspension containing 10^7 AM was distributed in four wells of a disposable plastic cluster dish. This sample volume was used to provide a sufficient number of AM required for the assays. After 21 hr incubation at 37°C, the viability of AM was determined and, after transfer into siliconized tubes, they were centrifuged at 365xg and washed in Hanks salt solution three times. The cell pellet was resuspended in distilled water for osmotic shock, the volume adjusted to 2.0 ml and the suspension cooled in ice was sonicated for a total of 45 sec in two bursts with the microtip of an ultrasonic generator using maximum setting. A portion of the cell suspension was used for enzyme assays after centrifugation at 63xg to remove the metal particles and a second portion was treated with sodium deoxycholate, the resulting lysate centrifuged at 10,000xg and the supernatant used for protein assay.

Enzyme activity was determined in triplicate using commercial assay kits (Boehringer Mannheim Corporation, Sigma Chemical Company). Acid phosphatase activity was determined by using the enzyme to hydrolyze p-nitro-phenylphosphate at 37°C, and measuring the liberated p-nitrophenol colorimetrically at 405 mμ. Beta-glucuronidase activity was measured by using phenolphtalein-glucuronic acid as a substrate and monitoring the liberated phenolphtalein colorimetrically at 550 mμ. LDH activity was analyzed by measuring the rate of oxidation of NADH at 366 mμ. This oxidation is proportional to the conversion by the enzyme of pyruvate to lactate. Total protein content was analyzed by the Lowry method (5) using a bovine serum albumin standard.

CALCULATION AND ANALYSIS OF DATA

Specific enzyme activities were calculated in mU/mg cellular protein. Changes in enzyme activity and in total cellular protein content were expressed as percentage of the corresponding controls. Since viability of the control AM was not affected by the 21 hr incubation, the viability values were reported directly as a percentage of total AM in the experimental sample. The data on AM viability, specific enzyme activity and total protein levels obtained in the dose response experiments were subjected to linear regression analysis.

SECTION 5

RESULTS AND DISCUSSION

VIABILITY AND PHAGOCYTOSIS

Viability of the macrophages was studied to establish the relative toxicity of the coated and uncoated fly ash in the three particle size ranges. When the cells were incubated in the presence of 50 to 1500 μg of particles per 10^6AM/ml, a close dose response relationship between the concentration of the particles and viability was observed. Within each particle size, decreased viability of AM was seen upon increase in concentration of the particles. This response appeared to be linear, generally providing a significant correlation coefficient (r) ranging from 0.906 to 0.995 for nine out of the twelve particles tested.

Figure 1 shows the least square lines of AM viability plotted for the various particles as well as the actual mean percent viability. The data indicate that, at a given concentration, AM viability decreased consistently with decrease in particle size. Although untreated fly ash reduced AM viability to a much lesser extent, nevertheless the particle size effect was clearly evident.

The estimated concentrations of particles that reduced the AM viability to 75% (LC_{75}) during a 21-hr incubation are shown in Table 1. The LC_{75} rather than the LC_{50} values were chosen to

TABLE 1. ESTIMATED CONCENTRATION OF PARTICLES REQUIRED TO REDUCE MACROPHAGE VIABILITY TO 75% DURING A 21-HR EXPOSURE

Particle Coating \ Size, μ	Particle Concentration, μg/10^6AM/ml		
	<2	2-5	5-8
PbO	180	270	470
NiO	320	550	870
MnO_2	375	510	860
None	870	1160	2700*

* Extrapolated value.

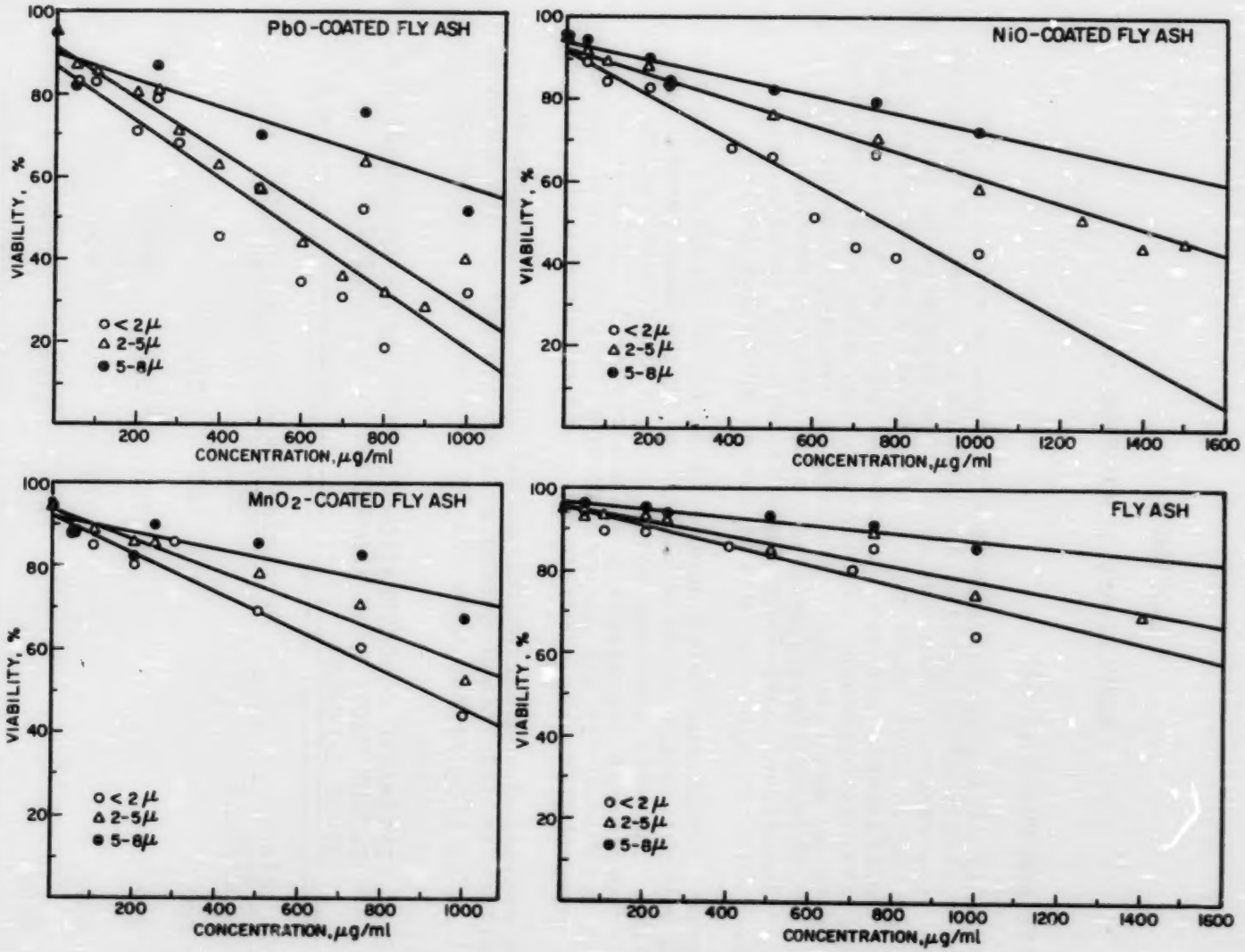

Figure 1. Effect of particle concentration and size on viability of macrophages. Each point represents the mean of multiple experiments.

establish the toxic ranking because in most instances the concentrations of particles that reduced AM viability to 50% were markedly higher than those actually included in the exposure experiments, thus would have to be determined by extrapolation. The results indicate that, irrespective of the particle size, PbO-coated fly ash was the most toxic with the NiO- and MnO_2-coated fly ash particles following close to each other. The uncoated fly ash particles reduced viability at markedly higher concentrations than the coated particles.

The effect of particle concentration and size on phagocytic activity was determined. The data indicate that the percentage of macrophages that had engulfed particles increased with increasing particle concentration and decreasing particle size. Moreover, in presence of the smaller particles not only more macrophages have phagocytized but also more particles were found within each cell. The data also show that, as expected, for a given particle size the percentage of phagocytizing AM was independent from the test compounds adsorbed on their surface. This is demonstrated in Figure 2 where the means of pooled observations of the four particle types within each size range are shown. In presence of the <2 μ particles, more than 90% of the AM phagocytized at the lowest concentration (250 μg/ml) and saturation was partially attained at the next highest concentration (500 μg/ml). For the intermediate and the largest particles the process was more gradual and more strongly concentration dependent. Also, for the 5-8μ particles, even at the highest concentration, only 85% of the AM phagocytized.

The amounts of metal elements deposited on the various fly ash particles were analyzed by atomic absorption spectrophotometry and the results are shown in Table 2.

TABLE 2. PERCENT OF METAL ELEMENTS ADSORBED ON FLY ASH PARTICLES

Coating	Metal Element, % Particle Size, μ		
	<2	2-5	5-8
PbO	3.85	3.84	3.15
NiO	2.63	2.91	2.77
MnO_2	3.22	3.67	2.91
None	a	a	a

[a] Uncoated fly ash contained <0.03% Pb, <0.03% Ni, and <0.01% Mn.

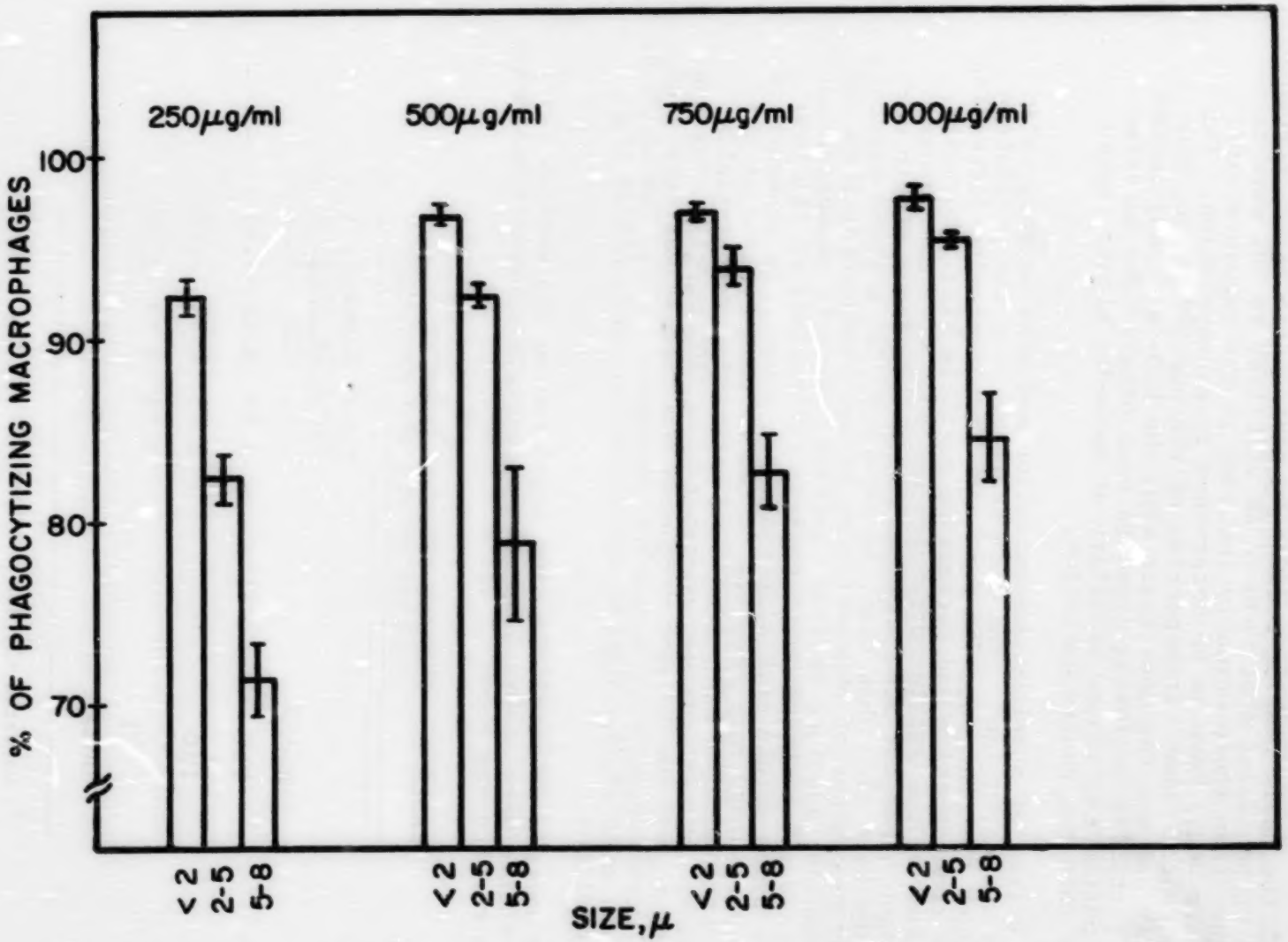

Figure 2. Effect of particle size and concentration on phagocytosis. Means + SE of observations for the combined four particle types within each size range are shown.

The percentage of adsorbed test metal varied within a relatively narrow range for all particles, and the variability within each particle type was even smaller. Therefore, on a weight/cell basis, the AM were exposed to fairly constant concentration of the test metals irrespective of particle size. The increased toxicity observed with the decreasing particle size, suggests that the toxic effect is due to concentration as well as to surface interaction between particles and alveolar macrophages. This is in agreement with the observations that, within a given concentration, the percentage of phagocytizing macrophages and the apparent number of particles engulfed by the AM increases with decreasing particle size. Thus, as smaller particles can be phagocytized easier in larger numbers, they can provide more surface area for interaction with the lysosomal membrane or release of cytotoxic components within the phagolysosomes. Large particles do not affect AM viability as readily because they cannot be engulfed in similar numbers and, after engulfment, a smaller surface area is available to interact with the lysosomal hydrolases.

Separate studies were conducted to determine whether the observed toxic effects were due to the solubilization of the metal element from the particles during the exposure. The test particles were incubated at 37°C for 21 hr and the particle suspension (P + S), the filtered particles resuspended in fresh maintenance medium (P), and the maintenance medium filtered through a 0.22 μ Millipore filter (S) were subsequently incubated with AM suspensions, and after 21 hr AM viabilities were determined. The results summarized in Table 3 indicate that viability of AM was equally affected by exposure to the original particle suspension or the incubated particles resuspended in fresh maintenance medium. The filtered culture media did not produce decreases in macrophage viability. This clearly demonstrates that the toxicity was not the result of solubilization of the test compounds in the medium.

TABLE 3. TESTING METAL OXIDE-COATED AND UNCOATED PARTICLES FOR SOLUBLE CYTOTOXIC COMPONENTS BY MACROPHAGE VIABILITY

Particle		AM Viability, %[a]		
Size, μ	Coating	P + Sb	Pb	Sb
<2	PbO	24.3	25.1	92.2
	NiO	33.3	31.2	91.5
	MnO_2	31.7	31.4	93.4
	None	35.2	35.8	92.6
2-5	PbO	22.5	26.7	90.9
	NiO	52.6	41.9	91.7
	MnO_2	37.3	33.2	92.7
	None	52.0	49.5	93.0
5-8	PbO	26.7	26.2	92.6
	NiO	66.2	58.6	92.0
	MnO_2	44.7	53.5	94.0
	None	80.1	81.3	92.0

[a] Viability of control AM = 94.0%

[b] Viability determined after exposure at 37°C for 21 hr at concentrations of 1000 μg/10^6AM/ml to

P + S Original particle suspension
P Particles separated from medium
S Particle-free filtered medium

ENZYME ACTIVITY

Activity of LDH, acid phosphatase, and β-glucuronidase and the levels of total cellular protein in AM after 21-hr incubation with the particles were studied to determine if a correlation with viability could be demonstrated or if a more sensitive indicator of cell dysfunction could be established. The PbO- and NiO-coated <2μ and 2 to 5μ particles were used with exposures to five concentrations for each particle type that resulted in viabilities ranging from 30 to 80%. The results (Figure 3) indicate that the decrease in viability was generally paralleled by decreases in cellular protein levels (representing cell lysis) and specific activity of LDH. A inconsistent decreasing trend could be observed for acid phosphatase and there appeared to be no change in the activity of β-glucuronidase.

The values shown in Figure 3 represent the means and standard error of three replicate experiments with triplicate assay determinations at each point. The considerably wider standard error in enzyme and occasionally in protein determinations compared to those of viability are the result of the complexity of the assay procedures. In addition, errors due to procedural factors introduced in the unexposed controls, which serve to calculate the percentage changes, could skew considerably the final specific enzyme activity levels calculated for the exposed samples. This is the reason for obtaining specific enzyme values exceeding the control levels, as we found to be the case frequently for β-glucuronidase and occasionally at lower concentrations for acid phosphatase and LDH.

The relationship between the concentration of the particles and decreases in viability, LDH activity and protein levels was linear (correlation coefficient ranging from 0.906 to 0.995). As can be seen from Table 4, the estimated concentrations of the particles that reduced these experimental parameters to 50% generally confirmed the toxic ranking determined in the viability-dose response experiments.

The PbO- and NiO-coated <2μ and 2 to 5μ particles at concentrations that resulted in 50% reduction of viability were compared with uncoated fly ash particles in the same particle size ranges and concentrations. Although all parameters were affected by the uncoated fly ash particles, the decreases produced were generally markedly smaller than upon exposure to the coated particles (Figure 4). The results of six replicate experiments demonstrated that the differences in viability and total protein levels were significant ($P < 0.001$); however, for enzyme activities this was not always the case.

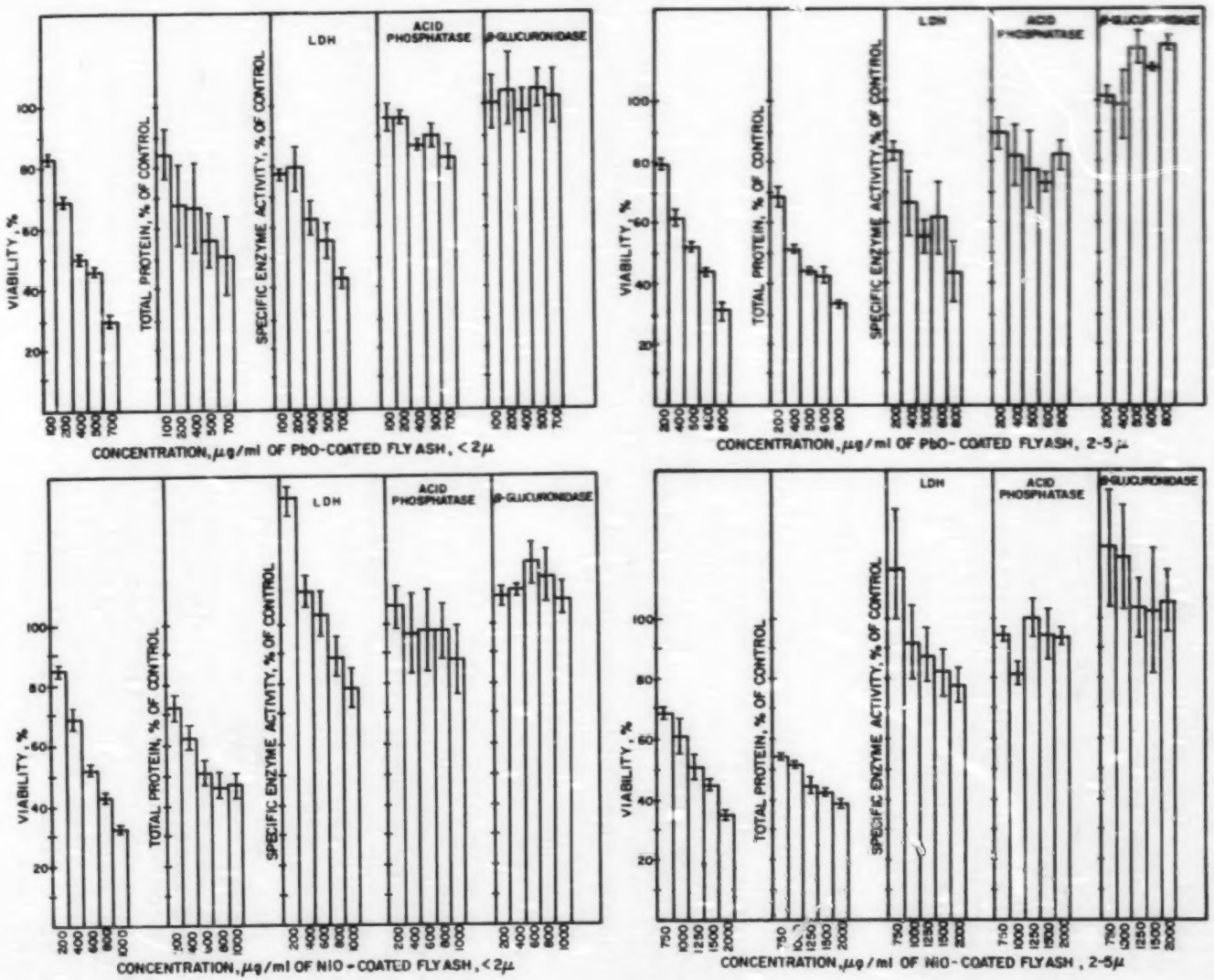
VIABILITY, %
TOTAL PROTEIN, % OF CONTROL
SPECIFIC ENZYME ACTIVITY, % OF CONTROL
LDH
ACID PHOSPHATASE
β-GLUCURONIDASE
CONCENTRATION, μg/ml OF PbO-COATED FLY ASH, < 2μ
CONCENTRATION, μg/ml OF PbO- COATED FLYASH, 2-5μ
CONCENTRATION, μg/ml OF NiO - COATED FLY ASH, < 2μ
CONCENTRATION, μg/ml OF NiO - COATED FLYASH, 2-5μ

Figure 3. Effect of particle exposure on macrophage viability, total protein content and specific enzyme activity. Means ± SE of three experiments are shown.

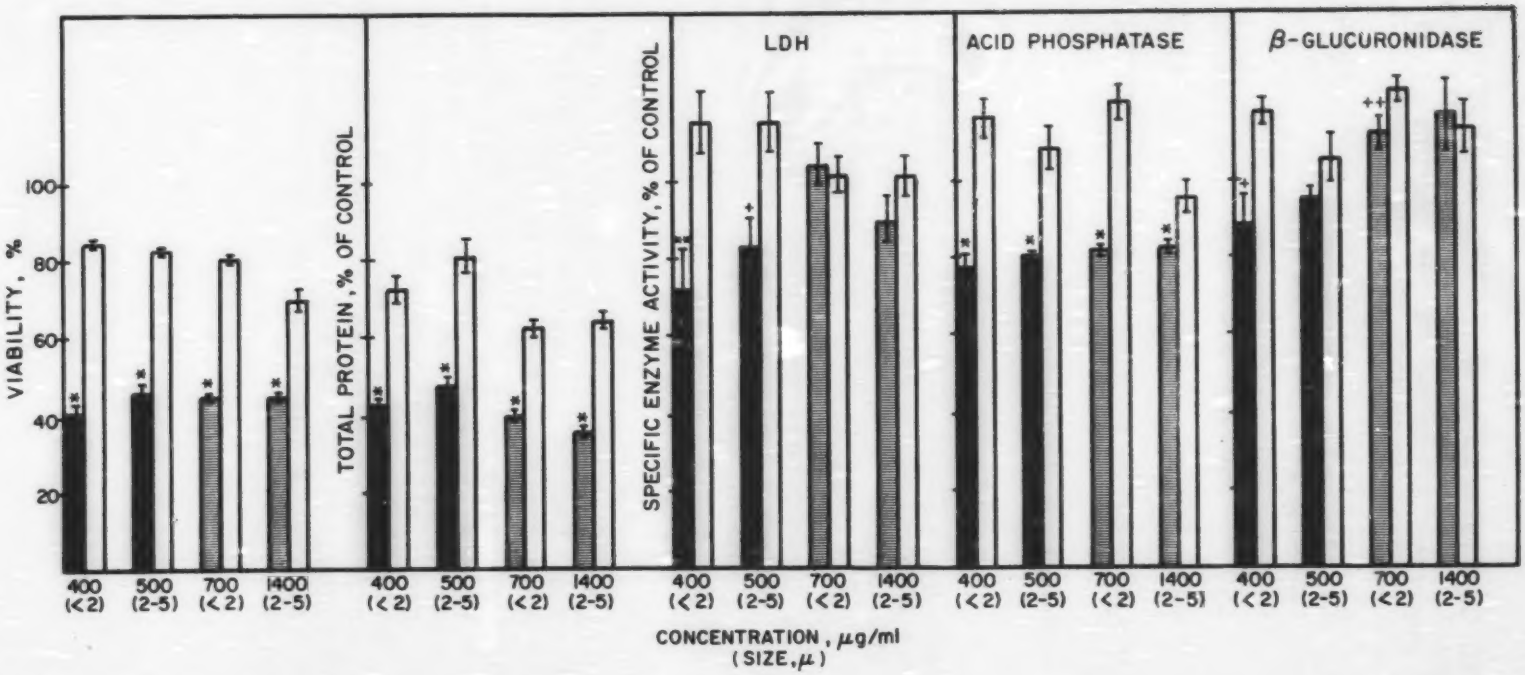

Figure 4. Comparison of the effects of PbO-coated (■) or NiO-coated (▩) and uncoated (□) fly ash on viability, total protein content and specific enzyme activity. Means + SE of six experiments are shown. Significance for the comparisons: * $P \leq 0.001$; ** $P \leq 0.01$; + $P \leq 0.025$; ++ $P \leq 0.1$.

TABLE 4. ESTIMATED CONCENTRATION OF PARTICLES REQUIRED TO REDUCE MACROPHAGE VIABILITY, TOTAL PROTEIN CONTENT AND LDH SPECIFIC ACTIVITY TO 50% DURING 21-HR EXPOSURE

Particle Coating\Size, μ	Particle Conc, μg/10^6 AM/ml, that Reduce to 50% Viability 2	Viability 2-5	Protein Content 2	Protein Content 2-5	LDH Activity 2	LDH Activity 2-5
PbO	450	560	670	480	485	700
NiO	685	1370	760	1100	1345*	3960*

* Above the tested concentration range.

Another experiment with these PbO- and NiO-coated and uncoated fly ash particles was conducted to determine if the presence of particles affected the enzyme assay system. The particles at the concentrations used in the experiments were added to AM preincubated for 21 hr at 37°C. The enzyme assay procedure, including all preparative steps, was immediately performed. The results showed no differences in the enzyme levels of these samples compared to control cells. Thus, there appeared to be no interference by the particles with any of the enzyme assays.

EFFECT OF TIME

Preliminary experiments were also conducted to investigate the effect on AM viability of length of incubation in presence of particles in the <2 μ size range. As shown in Table 5, no changes were observed during the first 5 hr of the incubation. After 14 hr, gradual decrease in viability was seen for all particles with the most rapid decrease observed in AM exposed to PbO-coated fly ash. In control AM, the viability remained constant throughout the 21-hr period. These observations suggest that the mechanism of action of these particles is similar to that described for silica where cell destruction starts after a lag time that permits sufficient hydrogen bonding of the lysosomal membranes to the silica in the phagolysosomes.

TABLE 5. EFFECT OF DURATION OF INCUBATION WITH PARTICLES[a] ON VIABILITY OF MACROPHAGES

Exposure Time, hr	Viability, %				
		Particle Coating			
	Control	None	PbO	NiO	MnO_2
1	97	94	94	95	96
3	97	94	96	95	97
5	98	96	97	94	96
14	97	88	76	74	87
15.5	96	84	62	80	83
18.5	95	71	46	56	66
20	97	60	32	48	57
21	96	70	40	55	53

[a] Estimated concentration of particles producing 50% reduction in AM viability in 21 hr. Particle size range: <2μ.

SECTION 6

RELATED STUDIES

Preliminary studies were conducted with cadmium oxide-(CdO) coated fly ash. The particles were prepared in<2, 2 to 5 and 5 to 8μ size ranges with respectively 12.1, 11.9 and 12.3% cadmium adsorbed on their surface. Cadmium was not detected by atomic adsorption spectrophotometry on the uncoated control fly ash particles.

In presence of 100 to 1000 μg particles/10^6AM/ml, viability of macrophages decreased between 50 to 60% even after exposure to the lowest concentrations. Results summarized in Table 6 indicate that this abrupt decrease in viability occurred in all three particle size ranges, with relatively little further change.

TABLE 6. EFFECT OF CdO-COATED FLY ASH PARTICLES ON MACROPHAGE VIABILITY[a]

Particle Concentration, μg/10^6AM/ml	Viability, %					
	CdO-Coated			Uncoated		
	<2μ	2-5μ	5-8μ	<2μ	2-5μ	5-8μ
100	58.4	59.9	50.1	90.2	92.7	95.4
250	49.3	46.6	44.0	89.5	95.2	92.7
300	53.4	47.1	55.0	80.5	85.7	91.8
500	54.3	40.0	42.9	85.3	89.0	94.0
600	49.6	48.7	42.7	73.9	74.8	90.9
750	51.6	33.4	34.0	81.1	87.7	91.5
900	37.5	36.9	28.1	65.8	63.1	88.3
1000	36.8	33.5	27.9	77.5	82.8	90.5

[a] Determined after 21 hr incubation at 37°C. Viability of control AM=96.7%.

Comparison with the uncoated fly ash showed that a further decrease in viability was generally observed at higher concentrations where the fly ash particles themselves produced a decrease in AM viability. These results strongly suggested that the toxicity of the test particles was partially due to solubilization of the CdO coating in the incubating medium. This was supported by visual observations in the light microscope of profuse bleb formation in the CdO-exposed AM, a phenomenon also seen under the scanning electron microscope on AM exposed *in vitro* to soluble cadmium salts (6).

To determine whether solubilization of the CdO coating was indeed taking place, CdO-coated test particles at the highest exposure concentration were incubated and AM were exposed to the filtered (0.22μ Millipore) medium. A viability decrease to approximately 50% was seen when AM were exposed to the filtered medium (S) confirming that solubilization has occurred (Table 7).

TABLE 7. TESTING OF CdO-COATED AND UNCOATED PARTICLES FOR SOLUBLE CYTOTOXIC COMPONENTS BY MACROPHAGE VIABILITY

Particle		AM Viability, %[a]		
Size, μ	Coating	P + S[b]	P[b]	S[b]
<2	None	49.9	67.6	94.8
	CdO	9.7	22.1	49.8
2-5	None	69.9	63.1	90.8
	CdO	20.9	37.6	50.2
5-8	None	77.1	67.8	82.7
	CdO	19.4	25.2	49.5

[a] Viability of control AM = 91.2%

[b] Viability determined after exposure at 37°C for 21 hr at concentrations of 1000 μg/10^6AM/ml to

P + S Original particle suspension
P Particles separated from medium
S Particle-free filtered medium

The viability of AM exposed to the original suspensions (P + S) was considerably lower since, in addition to the CdO that was released into the medium in the "presolubilization" period, the cells were also exposed to the whole system for an additional 21 hr. Solubilization of the CdO was also continuing since the resulting viability values were markedly lower than those shown in Table 6 for the corresponding exposure concentrations. According to this hypothesis, AM exposed to the preincubated washed particles (P) only should have intermediate viability values, which indeed was noted.

A preliminary experiment was conducted to monitor release of enzymes after incubation of the AM for 21 hr at 37°C with CdO-coated and uncoated fly ash particles (<2μ) at 500 μg/10^6AM/ml. The results indicated a marked decrease in acid phosphatase and β-glucuronidase activity in the CdO-exposed AM compared to the unexposed controls whereas LDH activity could no longer be detected after the CdO exposure. The AM exposed to uncoated fly ash showed little or no change in the activity of the three enzymes compared to the unexposed AM controls.

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